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Applicants	:	Christian Gr nhøj Larsen and Borbala Gesser
Serial no.	:	09/101,825
Filed	:	July 17, 1998
For	:	Synthetic IL-10 Analogues
Examiner	:	F. Hamud
Art unit	:	1646

Declaration of Borbala Gesser

1. I, Borbala Gesser, Pilegårdsvej 233, DK-8361 Hasselager, Kolt, Denmark, in my capacity as an assistant professor at Marselisborg Hospital, DK-8000 Aarhus C, Denmark, do state and declare as follows:

2. I am one of the named inventors of the above-captioned patent application. I believe that I am a person skilled in the art to which the above-captioned application pertains.

3. I have compiled the following information regarding the role of inflammatory cytokines in psoriasis and cancer. The information is derived from articles published in peer reviewed scientific journals as indicated by the list of references below as well as from work conducted in our laboratory. In doing so, my intention is to summarise what will be known to a person skilled in the art with respect to inflammatory cytokines in cancer and psoriasis.

4. Inflammatory cytokine´s effect on cancer: Inflammatory diseases are characterized by cells producing high concentration of inflammatory cytokines, specially IL-8, MCAF and TNF-α.
Cancer cells like melanomas produce IL-8, MCAF and serum levels of IL-8 correlate with the amount of tumour (Scheiber et al., 1995). Melanoma cells stimulated with IL-1α or TNF-α produced 10 fold higher quantities of IL-8 than normal human melanocytes (Zachariae et al., 1991).
IL-8 induces migration of melanoma cells (Ji Ming Wang et al., 1990) and IL-8 is involved in melanoma metastasis (Rakesh K. Sing et al., 1994).
Also human breast carcinoma cells respond chemotactically to IL-8 and MCP-1 and this chemokines might play a role in breast carcinoma cell migration (Sara J. Young et al., 1997).
Macrophages play a key role in inflammatory and tumour angiogenesis by releasing growth factors and monokines (IL-1, IL-6, IL-8, TNF-α, prostaglandins (Cord Sunderkøtter et al., 1994).

IL-10´s regulation of cancer: IL-10 release from transfected melanoma cells inhibited IL-1β, TNF-α, IL-6, proteinase matrix metalloproteinase-9 and vascular endothelial growth factor production from tumour associated macrophages. IL-10 thereby inhibited angiogenesis (Suyun Huang et al., 1996).
In a murine model of breast cancer IL-10 transfected tumour cells implanted subcutaneosly in immunocompetent BALB mice showed that tumour growth measured by

mean diameter was significantly inhibited, after 17 days, compared with non transfected cells (Namita Kundu et al., 1996).

IL-8´s effect on psoriasis: Interleukin-8 promotes epidermal cell proliferation Andrea Tuschil et al., 1992. It is also documented that psoriatic epidermis express high levels of IL-8 (Giustizieri M. L., et al., 2001).
Response of psoriasis to IL-10 is associated with suppression of IL-8/CXCR2 (IL-8 receptor) pathway and normalisation of keratinocyte maturation (Reich K et al., 2001).
Tumour suppresser protein p53 is found in decreased amount in involved psoriatic skin compared with uninvolved skin and normal skin. Antipsoriatic drug Tacrolimus was able to suppress IL-8 and induce p53 transcription (Michel et al., 1996).

The importance of suppressing inflammation in psoriasis or cancer was further proved by Hudson et al., 1999, who showed that " A pro-inflammatory cytokine (MIF) inhibits p53 tumour suppressor activity". In a variety of tumours, p53 is functionally inactivated, but the gene remains intact (Moll, U.M., 1992, 1995). Thus, identification and characterisation of novel regulators of p53 activity may have direct consequences for understanding the etiology of multiple tumour types.

To be able to address which type of cancer can be suppressed by IL-10 or IT9302, we investigated three Hepato-cellular carcinoma cells, HepG2 (p53 wild type), PLC/PRF (p53 mutated) and Hep3B (p53 deleted). Cells were exposed to high doses of IL-10 or IT9302 (μg/ml), relevant to cancer situation.

> See article Gesser et al., 2003, "The C-terminal part of IL-10 is regulating proliferation and apoptosis in hepato-cellular carcinoma cells through a p53 dependent IκB-α mechanism."

HepG2 cells, after daily stimulation with IT9302 (5μg/ml) or IL-10 (20μg/ml) showed inhibition of cell proliferation and induced apoptosis after 5 days of cell culture.
IL-8 was first induced day 2 accompanied by the induction of p53 at day 3. There was no induction of p53 in PLC/PRF5 cells and in Hep3B cells.
Relative induction of IL-8 secretion and relative cellular P53 protein expression was reversed from day three in HepG2 cells. See Fig. 4-6. IL-8 production was down regulated and p53 production was induced.

Regulation of IL-8 by IL-10 or IT9302 is significantly dependent on p53 wild type: P53 activity was also verified by induction of p21 protein in IT9302 stimulated cells compared with the non stimulated cells. Start of apoptosis was shown by PARP cleavage from day 4 and 5 see Fig. 12.
The transcription factor NF-κB controls the induction of several inflammatory genes like the IL-1β induction of IL-8 and NF-κB also controls apoptosis Ref.. The binding site of the p53 promoter contains response elements for NF-κB and the expression of p53 can be induced by NF-κB/p65 and TNF-α (Ref). We showed that IL-10 and IT9302 could regulate NF-κB binding to DNA in HepG2 cells, by using specific κB motif oligo-nucleotides from IL-8 and p53 promoter DNA Fig. 10 and 11.

NF-κB activation in inflammation is biphasic: DNA binding activity occurs once on the onset of inflammation, early, and later after 48 hours at the resolution of inflammation. Time study analysis of cytokine and apoptosis related gene expression in carrageenin-induced pleurisy showed that for example TNF-α was induced at 6 hours while bax (apoptosis regulator protein) reached maximum at 48 hours. Inhibition of NF-κB during the resolution

of inflammation protracted the inflammatory response and prevented apoptosis (Toby Lawrence et al., 2001). In conclusion, proper NF-κB regulation is essential for induction of apoptosis.

We next set out to investigate if any of the synthetic analogues were able to regulate NF-κB binding to DNA. We selected two IT9302 Synthetic analogues from the group of 35, which were tested for their ability to induce IRAP in purified monocytes.

Results from these experiments are collected in the following poster, which is enclosed with the present delaration:

> Novel Synthetic analogues of IL-10 regulate the binding of NF-kappaB complexes to p53 and IL-8 kapaB motifs. Christian Grønhøj Larsen, Claus Johansen, Lars Iversen, Arne Holm, Borbala Gesser Cytokines and Interferons 2002, Turin, October 6-10, 2002.

Conclusion: Il-10 as well as the IL-10 analogues significantly modulates the binding of NF-κB to DNA supporting the practicability of using the IL-10 analogues in the treatment of cancer. Furthermore, novel IL-10 analogues, which are modifications of IT9302 may have improved IL-10 like effects and may also exhibit enhanced stability compared to IT9302.

5. References:

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6. I further declare that all statements made herein of my own knowledge are true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: 13.07.03 Signature: Borbala Gesser

Borbala Gesser